METHOD FOR CONFIRMING HYDROGEN/DEUTERIUM EXCHANGE IN STRUCTURES OF BIOLOGICAL SAMPLES AND DETERMINING THE PERCENT CONTENT OF DEUTERIUM IN BIOLOGICAL SAMPLES

TECHNICAL FIELD

The invention relates to a method for analyzing hydrogen isotopes, especially a method for confirming hydrogen/deuterium exchange in the structure of a biological sample and determining the percent content of deuterium in the biological sample.

BACKGROUND ART

Since Urey *et al.* found deuterium, one of the hydrogen isotopes, in 1932, deuterium has been widely used in the biomedical field. The effect of heavy water on organisms, such as the effect of heavy water on the growth and development of bacteria, fungi, plants, mammal and the like, has been widely studied. In 1965, Mao Jiangsen, Huang Zhenxiang *et al.* found that Japanese encephalitis virus (JEV) which is extremely sensitive to heat has an increased heat stability when reproducing in cells treated with heavy water (Mao CS, Huang CH. Sci. Sin. 1965, 14(6): 885~890). In recent years, studies showed that poliomyelitis virus, influenza virus and hepatitis A virus (HAV) have a similar result when treated with heavy water (Wu R, Georgescu MM, Delpeyroux F. Vaccine, 1995, 13:1058~1063; and Ikizler MR, Wright PF. Vaccine, 2002, 20:1393~1399). However, the real mechanism about the effect of heavy water on these viruses is still unclear. Although exchange of hydrogen for deuterium in viral

structures may be the rational explanation, hitherto, there are no direct methods capable of confirming hydrogen/deuterium exchange in viral particles and determining deuterium content thereof. Thus, deuteration conditions of biological samples cannot be optimized, and processes of deuteration cannot be normalized.

Studies about the analysis methods of hydrogen isotopes have been carried out for a long time, and hitherto, there are many analysis methods, such as cryoscopic method, refractive index method, resistivity method, thermal electric conductivity method, infra-red spectrometry, neutron thermal energy spectrometry, gas chromatography, nuclear magnetic resonance (NMR) and chemical ionization mass spectrometry and the like (Abrams, S. A., Wong, W. W. (Book) New York CABI Publishing, 2003; and Platzner IT, Habfast K, Walder AJ. et al. (Book) New York John Wiley & Sons Ltd., 1997). Stable isotope deuterium tracer mass spectrometry is an analysis method that is widely used in the area of biomedical research, and is extensively used for the determination and identification of metabolic products, the studies about the mechanism of metabolic pathway, and the determination of the concentration of medicaments and metabolic products in organisms. In this method, man can determine the molecular composition of the substance to be tested and thereby know the property of the substance to be tested, as well as determine the content of the substance to be tested, by measuring the intensity of molecular ions or few characteristic fragment ions. This method does not require an intact mass spectrogram, i.e., it is only required to separate the interested compound, and then obtain a part of mass spectrogram using ion monitoring technique and

determine the ratio between labeled and unlabeled components. The accuracy of the ratio determined by this method is very low, and generally 1~10%, whereas the method has specificity and high sensitivity. Separation techniques that were developed in the recent twenty years, such as capillary separation technique, greatly improve the accuracy, sensitivity, selectivity and exclusivity of stable isotope deuterium tracer mass spectrometry, and thus gradually become the most important analysis methods in the area of biomedical research. However, these methods also have some limitations: (1) the test instruments used in these methods are mainly gas chromatograph-mass spectrometer (GC-MS) and liquid chromatograph-mass spectrometer (LC-MS), which are expensive; (2) highly pure medicaments labeled with stable isotope deuterium are obtained hardly, their amount is small and the cost of synthesis is high; and (3) the treatment of sample and the manipulation of testing and analyzing techniques are complex and time consuming.

^{2}H-NMR method is widely used in biochemistry and clinical medicine due to its advantages such as going deep into the interior of substance, not destroying the sample, and providing parameters, e.g. the molecular weight and structure of biological molecules (Armellin S, Brenna E, Fronza G, et al. Analyst. 2004 Feb, 129(2):130-3; and Roger O, Lavigne R, Mahmoud M, et al. J Biol Chem. 2004 Jun 11, 279(24):24923-8). The abundance of deuterium in nature is as low as about 150×10^{-6}, and its sensitivity is about 150×10^{-6} of that of hydrogen. The nuclear magnetic moment of deuteron is smaller,its spin quantum number is 1, the gyro-magnetic ratio of deuteron is lower, and thus the resonance frequency of deuteron and the

dispersity of spectral line are low. Therefore, the sensitivity of ^{2}H-NMR method is very low. Accordingly, in order to achieve satisfactorily a signal-to-noise ratio and an accuracy of determination, larger amount of sample and longer analysis time are required, and it is needed to purify the sample prior to determination.

In recent years, the rapidly developing life science raised a need to determine the content of deuterium in a biological sample. In some areas of life science, such as the research on JEV and HAV, in general, deuterium-rich biological samples can only be provided at a level of microgram. If it is required to confirm whether hydrogen/deuterium exchange has occurred in the whole viral particle and to determine the percent content of deuterium in the viral particle, all the existing analysis method for hydrogen isotopes cannot be directly used for the analysis of hydrogen isotopes in these biological samples.

CONTENTS OF THE INVENTION

The aim of present invention is to provide a novel analysis method for hydrogen isotopes in order to demonstrate whether hydrogen/deuterium exchange has occurred in a biological sample of interest and to calculate the percent content of deuterium in the biological sample. Further studies can ascertain the optimal percent content of deuterium in biological samples and the optimal deuteration conditions of biological samples, and thus improve the stability of biological samples, such as polypeptide. The percent content of deuterium in a deuterated biological sample is determined by the method, and the result shows that its percent content of

deuterium is increased. By other experiments, it is proved that the biological characters of this deuterated biological sample are markedly changed, and mainly, the heat resistance is markedly changed.

In first aspect, the present invention provides a method for confirming hydrogen/deuterium exchange in the structure of a biological sample, comprising the following steps:

(1) selecting a known natural protein as matrix, and formulating it with PBS to form a matrix solution having an appropriate concentration;

(2) subjecting a deuterated biological sample to be confirmed and a corresponding natural biological sample respectively to the following manipulations: diluting the sample obtained after purification with PBS to form a diluted sample having a desired concentration, mixing homogeneously the diluted sample with a suitable amount of the matrix solution followed by lyophilization to form a lyophilized mixed sample, sufficiently oxidizing the lyophilized mixed sample with oxidants through burning in order to oxidize the hydrogen in the mixed sample to water, reacting the resulting water after separation with zinc to generate hydrogen gas, and determining the $^{2}H/^{1}H$ ratio of the generated hydrogen gas with a gas isotope mass spectrometer;

(3) comparing the determined $^{2}H/^{1}H$ ratio of the generated hydrogen gas of the deuterated biological sample to be confirmed with that of the corresponding natural biological sample, wherein if the $^{2}H/^{1}H$ ratio of the generated hydrogen gas of the deuterated biological sample to be confirmed is significantly higher than that of

the corresponding natural biological sample, it is confirmed that hydrogen/deuterium exchange has occurred in the structure of the deuterated biological sample to be confirmed.

In one embodiment of the confirming method according to the present invention, said known natural protein is bovine serum albumin (BSA), egg albumin or lysozyme.

In one embodiment of the confirming method according to the present invention, said biological sample is a microorganism sample or a biomacromolecule sample. In its preferred embodiments, said biomacromolecule is a ribonucleic acid (RNA), a protein or a polypeptide.

In second aspect, the present invention provides use of the above-mentioned method for confirming hydrogen/deuterium exchange in the structure of a biological sample in confirming the close correlation between the stability and heat resistance of microorganisms or biomacromolecules and the deuterium/hydrogen ratio thereof.

In third aspect, the present invention provides a method for determining the percent content of deuterium in the structure of a biological sample, comprising the following steps:

(1) selecting a known natural protein as matrix, and formulating it with PBS to form a matrix solution having an appropriate concentration;

(2) diluting a biological sample obtained after purification with PBS to various concentrations, subjecting multiple sample solutions that have different concentrations but the same volume respectively to the following manipulations: mixing homogeneously the sample

solution with a suitable amount of the matrix solution followed by lyophilization to form a lyophilized mixed sample, sufficiently oxidizing the lyophilized mixed sample with oxidants through burning in order to oxidize the hydrogen in the mixed sample to water, reacting the resulting water after separation with zinc to generate hydrogen gas, and determining the $^2H/^1H$ ratio of the generated hydrogen gas with a gas isotope mass spectrometer; and

(3) according to a formula, calculating the percent content of deuterium in the biological sample by utilizing the above $^2H/^1H$ ratios of hydrogen gas determined for the multiple samples.

In one embodiment of the above-mentioned determination method, said step (3) is carried out as follows:

(a) plotting the determined $^2H/^1H$ ratios of the multiple samples (expressed with $\delta D_{SA\text{-}SMOW}$) against the amount of biological sample/amount of matrix protein (expressed with m_{bio}/m_{MAT}), and thus obtaining a straight line having a good linear correlation, $\delta D_{SA-SMOW} = k \cdot \frac{m_{bio}}{m_{MAT}} + \delta D_{MAT-SMOW}$, and educing the slope of the straight line, $k = \frac{500 \cdot C_{bio}^{D}}{R_{SMOW} \cdot C_{MAT}^{H}}$;

(b) calculating the theoretical percent content of ^1H in the matrix protein, C_{MAT}^{H}, according to the amino acid composition of the matrix protein;

(c) according to the slope k, calculating the percent content of deuterium in the biological sample as $C_{bio}^{D} = k \times R_{SMOW} \times C_{MAT}^{H} \times \frac{1}{500}$, wherein R_{SMOW} represents the $^2H/^1H$ ratio of SMOW and its value is 155.76×10^{-6}.

In one embodiment of the determination method according to the present invention, said known natural protein is bovine serum albumin (BSA), egg albumin or lysozyme.

In one embodiment of the determination method according to the present invention, said biological sample is a microorganism sample or a biomacromolecule sample. In its preferred embodiments, said biomacromolecule is a ribonucleic acid (RNA), a protein or a polypeptide.

In fourth aspect, the present invention provides use of the above-mentioned determination method in ascertaining the deuterium content of a biological sample having the optimal heat stability or ascertaining the optimal deuteration conditions of a biological sample.

In fifth aspect, the present invention provides use of the above-mentioned determination method in confirming the close correlation between the stability and heat resistance of a microorganism or a biomacromolecule and the deuterium content thereof.

A confirmation and determination method according to the present invention comprises the following steps: selecting a matrix, mixing homogeneously a prepared biological sample with a suitable amount of the matrix followed by lyophilization to form a lyophilized mixed sample, sufficiently oxidizing the lyophilized mixed sample with oxidants through burning in order to oxidize the hydrogen in the mixed sample to water, reacting the resulting water after separation with zinc to generate hydrogen gas. The $^{2}H/^{1}H$ ratio of the generated hydrogen gas is determined with a gas isotope mass spectrometer,

wherein the $^2H/^1H$ ratio of the sample is expressed with $\delta D_{SA\text{-}SMOW}$, i.e. the change value of deuterium/hydrogen ratio of the sample relative to deuterium/hydrogen ratio of the international standard – Standard Mean Ocean Water (SMOW). Comparison between the results determined from samples prepared from a deuterated biological sample and those of a corresponding natural biological sample is made so as to confirm whether hydrogen/deuterium exchange has occurred in the microorganism and biomacromolecule, and the percent content of deuterium in the biological sample is calculated using corresponding formula. Further studies can ascertain the optimal deuterium content of a biological sample or the optimal deuteration conditions, and confirm that the stability and heat resistance of a microorganism or biological molecule are closely correlative with the deuterium content thereof.

A specific method according to the present invention comprises selecting a known natural protein as matrix and subjecting each of purified biological samples to the following manipulations: diluting the sample with phosphate-buffered saline (PBS) to form a sample solution having a desired concentration, taking 0.1 ml of the sample solution and adding it to 0.5 ml of a matrix solution containing 5 mg known natural protein to form a mixed sample, placing then the mixed sample into a lyophil apparatus of Type GEL-1 for lyophilization to such an extent that the water content of the mixed sample is <3% after lyophilization (determined by Karl Fischer Titrator method), removing adsorbed water in the lyophilized mixed sample and CuO (500 mg) and V_2O_5 (50 mg) as oxidants at a temperature of 100°C for 15-30 minutes, and oxidizing the

lyophilized mixed sample with the oxidants by burning at 800-950°C for 15-30 minutes so that the sample is fully oxidized to CO_2, H_2O, NO_2 and so on after complete reaction, separating the water in the product and transferred it into a reaction tube for water sample, and then reacting the water with zinc at 400-450°C for 1.5-2.5 hours and thereby converting into hydrogen gas, determining the $^2H/^1H$ ratio of the hydrogen gas with a gas isotope mass spectrometer MAT-251.

The $\delta D_{SA\text{-}SMOW}$ value of the hydrogen isotope in sample is represented with the change of the deuterium/hydrogen ratio of sample relative to the deuterium/hydrogen ratio of SMOW, and is defined as follows:

$$\delta D_{SA\text{-}SMOW} = \frac{R_{SA} - R_{SMOW}}{R_{SMOW}} \times 10^3$$

wherein R_{SA} represents the $^2H/^1H$ ratio of sample; R_{SMOW} represents the $^2H/^1H$ ratio of SMOW (which value is 155.76×10^{-6}); only the integral value of $\delta D_{SA\text{-}SMOW}$ is generally adopted. From the $\delta D_{SA\text{-}SMOW}$ value of sample, the percent content of deuterium in the biological sample can be calculated.

A specific determination method according to the present invention comprises the following steps:

1. selecting a known natural protein as matrix, and formulated it with PBS to form a matrix solution having a concentration of 10 mg/ml.

2. after purification, diluting each of biological samples with PBS to form a diluted sample solution having a desired concentration.

3. taking 0.1 ml of the diluted solution of biological sample and

added it into 0.5 ml of the matrix solution having a concentration of 10 mg/ml, and then placing the mixed sample into a lyophil apparatus of Type GEL-1 for lyophilization.

4. removing adsorbed water in the lyophilized mixed sample and the oxidants CuO (500 mg) and V_2O_5 (50 mg) at a temperature of 100°C for 15-30 minutes, and then oxidizing the lyophilized mixed sample with the oxidants by burning at 800-950°C for 15-30 minutes so that after complete reaction the sample is fully oxidized to CO_2, H_2O, NO_2 and so on, separating the water in the product and transferred it into a reaction tube for water sample, and then reacting the water with zinc at 400 °C-450°C for 1.5-2.5 hours and thereby converting into hydrogen gas, determining the $^2H/^1H$ ratio of hydrogen gas with a gas isotope mass spectrometer MAT-251.

5. According to the $\delta D_{SA\text{-}SMOW}$ of sample, calculating the percent content of deuterium in the biological sample, wherein the specific calculation formulae are described as follows.

Step I, $$\delta D_{SA-SMOW} = \frac{R_{SA} - R_{SMOW}}{R_{SMOW}} \times 10^3 \qquad (1)$$

wherein R_{SA} represents the $^2H/^1H$ ratio of mixed sample, R_{SMOW} represents the $^2H/^1H$ ratio of SMOW and its value is 155.76×10^{-6}.

Step II, R_{SA} represents the $^2H/^1H$ ratio of mixed sample consisting of matrix protein and biological sample, wherein each of the parameters is showed in Table 1.

Table 1. The parameters of matrix protein and biological sample

Substance	Symbol	Amount Of substance (μg)	Percent Content of Hydrogen (%)	Percent Content of Deuterium (%)
matrix protein	MAT	m_{MAT}	C_{MAT}^{H}	C_{MAT}^{D}
biological sample	bio	m_{bio}	C_{bio}^{H}	C_{bio}^{D}

$$R_{SA}=\frac{\frac{1}{2}\left(m_{MAT}\cdot C_{MAT}^{D}+m_{bio}\cdot C_{bio}^{D}\right)}{m_{MAT}\cdot C_{MAT}^{H}+m_{bio}\cdot C_{bio}^{H}}\approx\frac{1}{2}\cdot\frac{C_{bio}^{D}}{C_{MAT}^{H}}\cdot\frac{m_{bio}}{m_{MAT}}+\frac{1}{2}\cdot\frac{m_{MAT}\cdot C_{MAT}^{D}}{m_{MAT}\cdot C_{MAT}^{H}}$$

$$=\frac{1}{2}\cdot\frac{C_{bio}^{D}}{C_{MAT}^{H}}\cdot\frac{m_{bio}}{m_{MAT}}+R_{MAT}\cdots\cdots\left(\because m_{MAT}\cdot C_{MAT}^{H}>>m_{bio}\cdot C_{bio}^{H}\right)\cdots\cdots(2)$$

Step III,

$$\delta D_{SA-SMOW}=\frac{R_{SA}-R_{SMOW}}{R_{SMOW}}\times10^{3}=\frac{\frac{1}{2}\cdot\frac{C_{bio}^{D}}{C_{MAT}^{H}}\cdot\frac{m_{bio}}{m_{MAT}}+R_{MAT}-R_{SMOW}}{R_{SMOW}}\times10^{3}$$

$$=\frac{500\cdot C_{bio}^{D}}{R_{SMOW}\cdot C_{MAT}^{H}}\cdot\frac{m_{bio}}{m_{MAT}}+\delta D_{MAT-SMOW}\qquad(3)$$

wherein R_{SMOW} is 155.76×10^{-6}, $\delta D_{MAT\text{-}SMOW}$ can be determined, C_{MAT}^{H} can be calculated via its amino acid composition, and C_{bio}^{D} of biological sample is an unknown constant.

Step IV, if k is defined as $k=\frac{500\cdot C_{bio}^{D}}{R_{SMOW}\cdot C_{MAT}^{H}}$, Formula (3) can be

transformed to

$$\delta D_{SA-SMOW} = k \cdot \frac{m_{bio}}{m_{MAT}} + \delta D_{MAT-SMOW} \qquad (4)$$

and thereby there is a linear correlation between $\delta D_{SA-SMOW}$ and $\frac{m_{bio}}{m_{MAT}}$.

The gas isotope mass spectrometry assisted by matrix has obvious advantages that: firstly, the result of determination is precise and has a good repeatability; secondly, the amount of sample used in this method is extremely small; thirdly, the determination method is simple and easy to manipulate, and has low cost; fourthly, there is a linear correlation between the value of $\delta D_{SA-SMOW}$ of sample and the amount of biological sample added, and thus the value of C^{D}_{bio}, i.e. the percent content of deuterium in biological sample, can be calculated from the slope k of straight line or directly via Formula (3).

Description of Figures

Fig. 1 shows the results of comparison experiments about the heat stability of JEVs cultured in different environments.

The deuterated JEV (cultured with 36% D_2O) and the non-deuterated JEV (cultured without D_2O) are inactivated by heat at 50°C, and the decrease in Plaque Forming Unit (PFU) titer is plotted against time. When the inactivation times are the same, the decrease in PFU titer of the deuterated JEV is smaller than that of the non-deuterated JEV. In Fig. 1, V_D represents the deuterated JEV, and V_H represents the non-deuterated JEV.

Fig. 2 shows the results of comparison experiments about the stability of HAVs cultured in different environments.

The deuterated HAV (HAV particle resuspended in 87% D_2O)

and the non-deuterated HAV (HAV particle resuspended in H_2O) are stayed at 10°C, and the decrease in Medial Cell Culture Infectious Dose ($CCID_{50}$) titer is plotted against time. When the standing times are the same, the decrease in $CCID_{50}$ titer of the deuterated HAV is smaller than that of the non-deuterated HAV. In Fig. 2, V_D represents the deuterated HAV, and V_H represents the non-deuterated HAV.

Fig. 3 shows the $\delta D_{SA\text{-}SMOW}$ - m_{bio}/m_{BSA} graph of V_D sample prepared from the deuterated HAV.

Fig. 4 shows the $\delta D_{SA\text{-}SMOW}$ - m_{bio}/m_{BSA} graph of RNA-V_D sample prepared from ribonucleic acid (RNA) of the deuterated HAV.

Fig. 5 shows the $\delta D_{SA\text{-}SMOW}$ - m_{bio}/m_{BSA} graph of the deuterated bovine serum albumin (BSA) sample.

MODE OF CARRYING OUT THE INVENTION

The following non-limiting examples are intended to explain the present invention in more detail, but not to limit the scope of the present invention in any way.

Example 1

Confirmation of hydrogen/deuterium exchange in the whole viral particle of JEV

The primary chick embryo cells (CECs) were used to reproduce JEV. Firstly, CECs were allowed to grow in a culture medium without heavy water (D_2O) for 24h, and then were cultured in a maintenance medium containing 36% D_2O at 37°C for 2 days. The maintenance medium containing heavy water was discarded, and JEV was added into

the cells and adsorbed for 2h. After adsorption, the adsorption solution was discarded, the cells were washed twice with PBS, and then a fresh maintenance medium containing D_2O was added, and the resulting deuterated JEV (represented with V_D) was harvested after 3 days. In above-mentioned culture process, when CECs were cultured with a culture medium without D_2O and infected with JEV, a non-deuterated JEV (represented with V_H) was obtained. In above-mentioned culture process, when CECs were cultured with a culture medium containing D_2O but were not infected with JEV, a deuterated cell control without virus (represented with C_D) was obtained. All samples of virus and the corresponding cell control without virus were purified by precipitation with polyethylene glycol (PEG), filtration with 0.45 μm filter, ultracentrifugation with 40% sucrose underlaid, and the like. The virus samples were inactivated with formaldehyde, and then their titers were determined by hemagglutination method and expressed with Hemagglutination Unit (HAU).

It was found that the decrease in PFU titer of the deuterated JEV and that of the non-deuterated JEV varied with the inactivation time at 50°C, and the decrease in PFU titer of the non-deuterated JEV was greater than that of the deuterated JEV under the same inactivation time (see Fig. 1). This indicated that JEV reproduced in the presence of D_2O had an increased heat resistance.

BSA was selected as matrix. After purification, the deuterated JEV, the non-deuterated JEV and the deuterated cell control without virus were subjected respectively to the following manipulations: it was diluted with PBS to form a diluted sample solution having a desired concentration, 0.1 ml of the diluted sample solution was

taken and added into 0.5 ml of a PBS solution containing 5 mg BSA. Then, the resulting mixed sample was placed into a lyophil apparatus of Type GEL-1 for lyophilization to the extent that the water content of the mixed sample was <3% after lyophilization. The resulting samples were represented with V_D, V_H and C_D, respectively. Adsorbed water in the lyophilized mixed sample and the oxidants CuO (500 mg) and V_2O_5 (50 mg) was removed at a temperature of 100°C for 20 minutes, and then the oxidation reaction of the lyophilized mixed sample with the oxidants was carried out by burning at 850°C for 30 minutes so that after complete reaction the sample was fully oxidized to carbon dioxide (CO_2), water (H_2O), nitrogen dioxide (NO_2) and so on. The water in the product was separated and transferred into a reaction tube for water sample, and then the water was reacted with zinc at 420°C for 2 hours and thereby converted into hydrogen gas, the $^2H/^1H$ ratio of which was determined with a gas isotope mass spectrometer MAT-251. The determined values of $\delta D_{SA\text{-}SMOW}$ of samples V_D, V_H and C_D were shown in Table 2.

Table 2. The values of $\delta D_{SA\text{-}SMOW}$ of samples V_D、V_H and C_D

Sample	$\delta D_{SA\text{-}SMOW}$			
	Sample (μg)			
	0	5	10	20
V_D	-76±3	-35±3	-8±4	42±3
V_H		-72±2	-54±0	-60±0
C_D		-75±3	-65±0	-70±7

Because the value of $\delta D_{SA\text{-}SMOW}$ of the matrix BSA was a constant value, the values of $\delta D_{SA\text{-}SMOW}$ of the mixed samples reflected the levels of $^2H/^1H$ ratio of the added biological samples, and reflected the added amounts thereof. When the added amounts were the same, a larger value of $\delta D_{SA\text{-}SMOW}$ of the mixed sample indicated a higher $^2H/^1H$ ratio of the biological sample; when the added biological samples were from the same source, a larger value of $\delta D_{SA\text{-}SMOW}$ of the mixed sample indicated a larger added amount of the biological sample.

In Table 2, it can be seen that the value of $\delta D_{SA\text{-}SMOW}$ of sample V_D was larger than that of sample V_H when the added amounts of JEV were the same; and the value of $\delta D_{SA\text{-}SMOW}$ of sample V_D increased with the increase in the added amount of virus. The increase in $\delta D_{SA\text{-}SMOW}$ of sample V_D was unlikely to be obtained from the possible contaminating cellular components during the preparation of viral sample, because the values of $\delta D_{SA\text{-}SMOW}$ of sample C_D, the cellular components control without virus, were similar at different dosages, and ranged from -65 to –75, there was no positive correlation with the dosage, and the $\delta D_{SA\text{-}SMOW}$ of sample C_D was relatively close to that of the matrix BSA. This indicated that the deuterium in sample C_D, the cellular components control, was under the natural state. Therefore, compared with JEV obtained in the culture medium without D_2O, the $^2H/^1H$ ratio of the JEV reproduced in the presence of D_2O was increased significantly, i.e., the $^2H/^1H$ ratio of the deuterated JEV was higher than that of the non-deuterated JEV. This indicated that deuterium was enriched in the JEV reproduced in the presence of D_2O, i.e. the deuterium content thereof was increased, and that deuterium atoms replaced some hydrogen atoms in the JEV

particle.

Example 2

Confirmation of hydrogen/deuterium exchange in whole viral particle of HAV and determination of the percent content of deuterium thereof

The vaccine strain of hepatitis A virus (Strain H2) was reproduced in and obtained from the diploid cells of human lung. The cells infected with Strain H2 and the non-infected cells, the control, were harvested, and then resuspended with PBS, and were treated three times with freeze-thaw and ultrasonication, and then centrifugated at 8000 g for 30 minutes to remove the cell debris. The obtained supernatant was extracted three times with chloroform, and then the virus or the cell control was harvested by ultracentrifugation. The deuteration of sample was carried out as follows: the sample was resuspended in a minimum essential medium (MEM) containing 87% heavy water, and incubated at 36°C for one week. The deuterated sample was subjected to ultracentrifugation, the MEM containing heavy water was discarded, and the precipitate was washed well with distilled water and then ultracentrifugated again to harvest the deuterated sample. The washing process was repeated three times to ensure that the deuterated sample almost did not contain free heavy water. The $CCID_{50}$ titer of viral sample was determined by a cell culture method.

It was found that the decrease in $CCID_{50}$ titer of the deuterated HAV and that of the non-deuterated HAV varied with the standing time at 10°C, and the decrease in $CCID_{50}$ titer of the non-deuterated

HAV was greater than that of the deuterated HAV under the same standing time (see Fig. 2). This indicated that HAV incubated in the presence of D_2O had an increased stability.

BSA was selected as matrix. The deuterated HAV, the non-deuterated HAV and the deuterated cell control were subjected respectively to the following manipulations: it was diluted with PBS to form a diluted sample solution having a desired concentration, 0.1 ml of the diluted sample solution was taken and added into 0.5 ml of PBS solution containing 5 mg BSA. Then, the mixed sample was placed into a lyophil apparatus of Type GEL-1 for lyophilization to such an extent that the water content of the mixed sample was <3% after lyophilization. The resulting samples were represented with V_D, V_H and C_D, respectively. Adsorbed water in the lyophilized mixed sample and the oxidants CuO (500 mg) and V_2O_5 (50 mg) was removed at a temperature of 100°C for 20 minutes, and then the oxidation reaction of the lyophilized mixed sample with the oxidants was carried out by burning at 850°C for 30 minutes so that after complete reaction the sample was fully oxidized to carbon dioxide (CO_2), water (H_2O), nitrogen dioxide (NO_2) and so on. The water in the product was separated and transferred into a reaction tube for water sample, and then the water was reacted with zinc at 420°C for 2 hours and thereby converted into hydrogen gas, the $^2H/^1H$ ratio of which was determined with a gas isotope mass spectrometer MAT-251.

The $\delta D_{SA\text{-}SMOW}$ values of sample V_D prepared from the deuterated HAV were determined. The significant increase in $\delta D_{SA\text{-}SMOW}$ value of sample V_D cannot be resulted from the free water in the deuterated

HAV particle, because the $\delta D_{SA\text{-}SMOW}$ value of the last wash solution (SV) was consistent with that of distilled water; and this cannot be resulted from the possible contaminating deuterated cellular components during the preparation of viral sample neither, because the $\delta D_{SA\text{-}SMOW}$ value of sample C_D was at the background level. Therefore, the significant increase in $\delta D_{SA\text{-}SMOW}$ value of sample V_D indicated that the $^2H/^1H$ ratio of HAV was increased after incubation in D_2O, and the hydrogen/deuterium exchange occurred in the HAV particles and deuterium was enriched in them. Furthermore, the $\delta D_{SA\text{-}SMOW}$ value of sample V_D increased linearly with the increase in the added amount of deuterated HAV, i.e., the $\delta D_{SA\text{-}SMOW}$ value of sample V_D was directly proportional to the added amount of deuterated HAV within a defined range. The relation between the $\delta D_{SA\text{-}SMOW}$ value of sample V_D and the added amount of deuterated HAV was shown in Table 3.

Table 3. The relation between the $\delta D_{SA\text{-}SMOW}$ of sample V_D prepared from deuterated HAV and the added amount of deuterated HAV

Amount of the deuterated HAV (m_{bio}, μg)	Amount of the matrix BSA (m_{BSA}, μg)	m_{bio}/m_{BSA}	$\delta D_{SA\text{-}SMOW}$
0	5000	0	-88
0.2		0.04×10^{-3}	-51
0.5		0.10×10^{-3}	22
1		0.20×10^{-3}	55
2		0.40×10^{-3}	291

The $\delta D_{SA\text{-}SMOW}$ was plotted against m_{bio}/m_{BSA} (see Fig. 3).

It can be known from Fig. 3 that R^2 of the straight line was 0.9762, and thus there was a good linear correlation. The slope of the straight line was $k = \frac{500 \cdot C^D_{bio}}{R_{SMOW} \cdot C^H_{BSA}} = 917594$, and the intercept was $-90.00 = \delta D_{BSA\text{-}SMOW}$, which was consistent with the determined value for BSA, $\delta D_{BSA\text{-}SMOW} = -88$.

Calculation of the percent content of deuterium in the deuterated HAV

(a) The amino acid composition of BSA was known from literatures, and the total number of hydrogen atoms was calculated as 4614 and was then divided by 66408.77, the theoretical molecular weight of BSA, and thereby the theoretical percent content of ^1H was obtained as $C^H_{BSA} = 6.95\%$.

(b) According to the slope of the straight line, the percent content of deuterium in the deuterated HAV was calculated as

$$C^D_{bio} = 917594 \times R_{SMOW} \times C^H_{BSA} \times \frac{1}{500}$$

$$= 917594 \times 155.76 \times 10^{-6} \times 6.95\% \times \frac{1}{500}$$

$$= 1.99\%$$

Example 3

Confirmation of hydrogen/deuterium exchange in the ribonucleic acid (RNA) of deuterated HAV and determination of the percent content of deuterium thereof

The well-washed deuterated HAV or cell control was used to prepare the deuterated RNA, and the method referred to one-step

extraction method with guanidine isothiocyanate-phenol. RNA sample was quantified by spectrophotometry, and the OD_{260}/OD_{280} ratio was calculated to characterize the purity of RNA.

BSA was selected as matrix. The RNAs extracted from the deuterated HAV, the non-deuterated HAV and the deuterated cell control were subjected respectively to the following manipulations: the RNA was diluted with PBS to form a diluted sample solution having a desired concentration, 0.1 ml of the diluted sample solution was taken and added into 0.5 ml of PBS solution containing 5 mg BSA. Then, the mixed sample was placed into a lyophil apparatus of Type GEL-1 for lyophilization to such an extent that the water content of the mixed sample was <3% after lyophilization. The resulting samples are represented with RNA-V_D, RNA-V_H and RNA-C_D, respectively. Adsorbed water in the lyophilized mixed sample and the oxidants CuO (500 mg) and V_2O_5 (50 mg) was removed at a temperature of 100°C for 20 minutes, and then the oxidation reaction of the lyophilized mixed sample with the oxidants was carried out by burning at 850°C for 30 minutes, after complete reaction the sample was fully oxidized to carbon dioxide (CO_2), water (H_2O), nitrogen dioxide (NO_2) and so on. The water in the product was separated and transferred into a reaction tube for water sample, and then the water was reacted with zinc at 420°C for 2 hours and thereby converted into hydrogen gas, the $^2H/^1H$ ratio of which was determined with a gas isotope mass spectrometer MAT-251.

The values of $\delta D_{SA\text{-}SMOW}$ of samples RNA-V_D and RNA-V_H were determined. The results showed that the RNA of deuterated HAV has a relatively high deuterium content, and the value of $\delta D_{SA\text{-}SMOW}$ of

RNA-V_D increased with the increase in the amount of RNA added (see Table 4). Whereas, the deuterium content of the non-deuterated viral sample RNA-V_H or the deuterated cell control sample RNA-C_D was extremely low, and this indicated that the increase in $\delta D_{SA\text{-}SMOW}$ value of sample RNA-V_D was due to the high $^2H/^1H$ ratio in the added RNA of deuterated HAV, and the increase in $^2H/^1H$ ratio in the RNA of deuterated HAV was due to the exchange of hydrogen by deuterium.

Table 4. The relation between the $\delta D_{SA\text{-}SMOW}$ of sample RNA-V_D and the added amount of deuterated RNA

Amount of the deuterated RNA (m_{bio}, μg)	Amount of the matrix BSA (m_{BSA}, μg)	m_{bio}/m_{BSA}	$\delta D_{SA\text{-}SMOW}$
0	5000	0	-85
1		0.2×10^{-3}	-60
2		0.4×10^{-3}	-52
3		0.6×10^{-3}	-36
4		0.8×10^{-3}	-17
10		2×10^{-3}	86

The $\delta D_{SA\text{-}SMOW}$ was plotted aganist m_{bio}/m_{BSA} (see Fig. 4).

It can be known from Fig. 4 that R^2 of the straight line is 0.9965, and thus there was a good linear correlation. The slope of the straight line was $k = \frac{500 \cdot C^D_{bio}}{R_{SMOW} \cdot C^H_{BSA}} = 84211$, and the intercept was $-83.474 \approx \delta D_{BSA\text{-}SMOW}$, which is consistent with the determined value for BSA, $\delta D_{BSA\text{-}SMOW} = -85$.

Calculation of the percent content of deuterium in the RNA of deuterated HAV

(a) The amino acid composition of BSA was known from literatures, and the total number of hydrogen atoms was calculated as 4614 and then divided by 66408.77, the theoretical molecular weight of BSA, and thereby the theoretical percent content of ^1H was obtained as $C_{BSA}^{H} = 6.95\%$.

(b) According to the slope of the straight line, the percent content of deuterium in the RNA of deuterated HAV was calculated as

$$C_{bio}^{D} = 84211 \times R_{SMOW} \times C_{BSA}^{H} \times \frac{1}{500}$$

$$= 84211 \times 155.76 \times 10^{-6} \times 6.95\% \times \frac{1}{500}$$

$$= 0.18\%$$

Example 4

Determination of the percent content of deuterium in the deuterated bovine serum albumin (BSA) sample

The deuteration of BSA was carried out as follows: 500 mg of BSA was diluted in 10 ml of phosphate-buffered saline (PBS) containing 90% heavy water, and thereto the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM, and the bacteriostat sodium azide was added to a final concentration of 0.2 mg/ml, and then the resulting mixture was incubated at 20°C for one week. The deuterated BSA was dialyzed against 1000 ml of PBS, and the solution was changed four times in order to completely remove the free heavy water. The deuterated BSA sample obtained after the last dialysis was

determined for its protein concentration by spectrophotometry.

BSA was selected as matrix. The deuterated BSA was diluted with PBS to form a diluted sample solution having a desired concentration, 0.1 ml of the diluted sample solution was taken and added into 0.5 ml of PBS solution containing 5 mg BSA. Then, the mixed sample was placed into a lyophil apparatus of Type GEL-1 for lyophilization to such an extent that the water content of the mixed sample was <3% after lyophilization, and thereby the deuterated BSA sample to be tested was obtained. Adsorbed water in the lyophilized mixed sample and the oxidants CuO (500 mg) and V_2O_5 (50 mg) was removed at a temperature of 100°C for 20 minutes, and then the oxidation reaction of the lyophilized mixed sample with the oxidants was carried out by burning at 850°C for 30 minutes so that after complete reaction the sample was fully oxidized to carbon dioxide (CO_2), water (H_2O), nitrogen dioxide (NO_2) and so on. The water in the product was separated and transferred into a reaction tube for water sample, and then the water was reacted with zinc at 420°C for 2 hours and thereby converted into hydrogen gas, the $^2H/^1H$ ratio of which was determined with a gas isotope mass spectrometer MAT-251.

The $\delta D_{SA\text{-}SMOW}$ value of the deuterated BSA sample was determined. The results showed that the deuterated BSA had an increased content of deuterium (see Table 5).

Table 5. The relation between the $\delta D_{SA\text{-}SMOW}$ of the deuterated BSA sample and the added amount of the deuterated BSA

Amount of the deuterated BSA (m_{bio}, μg)	0	30	50	70	90
Amount of the matrix BSA (m_{BSA}, μg)	5000				
m_{bio}/m_{BSA}	0	6×10^{-3}	10×10^{-3}	14×10^{-3}	18×10^{-3}
$\delta D_{SA\text{-}SMOW}$	-116	-92	-70	-52	-39

The $\delta D_{SA\text{-}SMOW}$ was plotted against m_{bio}/m_{BSA} (see Fig. 5).

It can be known from Fig. 5 that R^2 of the straight line was 0.9947, and thus there was a good linear correlation. The slope of the straight line was $k = \frac{500 \cdot C^D_{bio}}{R_{SMOW} \cdot C^H_{BSA}} = 4408$, and the intercept was $-116.11 = \delta D_{BSA\text{-}SMOW}$, which was consistent with the determined value for BSA, $\delta D_{BSA\text{-}SMOW} = -116$.

Calculation of the percent content of deuterium in the deuterated BSA

(a) The amino acid composition of BSA was known from literatures, and the total number of hydrogen atoms was calculated as 4614 and then divided by 66408.77, the theoretical molecular weight of BSA, and thereby the theoretical percent content of ^1H was

obtained as $C_{BSA}^{H} = 6.95\%$.

(b) According to the slope of the straight line, the percent content of deuterium in the deuterated BSA was calculated as

$$C_{bio}^{D} = 4408 \times R_{SMOW} \times C_{BSA}^{H} \times \frac{1}{500}$$

$$= 4408 \times 155.76 \times 10^{-6} \times 6.95\% \times \frac{1}{500}$$

$$= 0.01\%$$